EXHIBIT 7

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Chromosome-selective sequencing of maternal plasma cell-free DNA for first-trimester detection of trisomy 21 and trisomy 18

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Condensation

Chromosome-selective sequencing of maternal cfDNA and fetal-fraction-optimized data analysis provide effective first-trimester detection of fetal trisomies 21 and 18.

Short version of article title

Chromosome-selective sequencing for non-invasive detection of trisomies 21 and 18

Abstract

Objective: To assess the prenatal detection rate of trisomies 21 and 18 and false positive rate by chromosome-selective sequencing of maternal plasma cell-free DNA (cfDNA).

Study design: Nested case-control study of cfDNA was examined in plasma obtained before chorionic villous sampling from 300 euploid, 50 trisomy 21 and 50 trisomy 18 pregnancies at 11-13 weeks. Laboratory personnel were blinded to fetal karyotype.

Results: Risk scores for trisomy 21 and trisomy 18 were given for 397 of the 400 samples that were analyzed. In all 50 cases of trisomy 21 the risk score for trisomy 21 was ≥99% and the risk score for trisomy 18 was ≤0.01%. In all 50 cases of trisomy 18 the risk score for trisomy 21 was ≤0.01% and the risk score for trisomy 18 was ≥99% in 47 cases, 98.8% in one, 88.5% in one and 0.11% in one. In 3 (1%) of the 300 euploid pregnancies no risk score was provided because there was failed amplification and sequencing. In the remaining 297 the risk score for trisomy 21 was ≤0.01% and the risk score for trisomy 18 was ≤0.01% in 295 cases, 0.04% in one and 0.23% in one. Therefore, the sensitivity for detecting trisomy 21 was 100% (50/50), the sensitivity for trisomy 18 was 98% (49/50) and the specificity was 100% (297/297).

Conclusion: In this study, chromosome-selective sequencing of cfDNA separated all cases of trisomy 21 and 98% of trisomy 18 from euploid pregnancies.

Key words: Non-invasive prenatal diagnosis; Trisomy 21; Trisomy 18; First trimester.

Introduction

Diagnosis of fetal aneuploidies relies on invasive testing, by chorionic villous sampling or amniocentesis, in pregnancies identified by screening to be at high risk for such aneuploidies [1]. In the 1970s and 1980s, the main method of screening for aneuploidies was by maternal age with a cut-off of 35 years to define the high-risk group. This was associated with a 5% screen-positive rate and a detection rate of trisomy 21 of 30%. In the late 1980s and 1990s screening was provided by a combination of maternal age and serum biochemistry in the second-trimester, which resulted in improvement of the detection rate to 50-70% with the same 5% screen positive rate. In the last 15 years the emphasis of screening shifted to the first-trimester where a combination of maternal age, fetal nuchal translucency (NT) thickness and maternal serum free ß-human chorionic gonadotrophin (ß-hCG) and pregnancy associated plasma protein-A (PAPP-A) could identify about 90% of fetuses with trisomies 21, 18 and 13 [2-3]. In specialist fetal medicine centres addition of other first trimester sonographic markers, including the nasal bone and Doppler flow in the ductus venosus, hepatic artery and across the tricuspid valve could improve the detection rate of aneuploidies to more than 95% and reduce the screen positive rate to less than 3% [1,4].

Recently, non-invasive prenatal detection of fetal aneuploidies has been achieved by exploiting the presence of cell free DNA (cfDNA) in maternal plasma [5]. In trisomy 21 compared to euploid pregnancies the amount of chromosome 21 in maternal plasma is slightly higher than that of other chromosomes because there are three rather than two copies of fetal chromosome 21. Massively parallel shotgun sequencing

(MPSS), which can identify and quantify millions of DNA fragments, has now made it possible to detect the increment in chromosome 21 in the plasma of affected pregnancies [6,7]. Using this approach trisomy 21 and to a lesser extent trisomy 18 has been successfully detected non-invasively [8-13]. Essentially, maternal plasma DNA molecules are sequenced and the chromosomal origin of each molecule is identified by comparing to the human genome. In trisomy 21 pregnancies the number of molecules derived from chromosome 21, as a proportion of all sequenced molecules, is higher than in euploid pregnancies. However, this approach requires a significant amount of DNA sequencing which can be costly and has a limited throughput. Since MPSS is not selective in the chromosomal origin of the sequenced DNA fragments and chromosome 21 represents only about 1.5% of the human genome it is necessary to sequence many millions of fragments to ensure sufficient chromosome 21 counts. An alternative to MPSS that may overcome these limitations is selective sequencing of loci from only chromosomes under investigation. Such chromosome-selective sequencing, referred to as digital analysis of selected regions (DANSR), has been successfully applied to the non-invasive detection of trisomies 21 and 18 [14]. Sparks *et al.,* have introduced the fetal-fraction optimized risk of trisomy evaluation (FORTE) by extending the process of chromosome-selective sequencing to assay non-polymorphic and polymorphic loci, where fetal alleles differ from maternal alleles, enabling simultaneous determination of chromosome proportion and fetal fraction [14].

The objective of this study is to assess the prenatal detection rate of trisomies 21 and 18 and false positive rate at 11-13 week's gestation by the DANSR assay and the FORTE algorithm.

Materials and Methods

Study population

This was a nested case-control study of stored maternal plasma from 400 singleton pregnancies at 11-13 weeks' gestation, including 300 with euploid fetuses, 50 with trisomy 21 and 50 with trisomy 18. In all cases fetal karyotyping was carried out by chorionic villous sampling in our tertiary referral centre because screening by the combined test in their hospital demonstrated that the risk for aneuploidies was more than 1 in 300. Gestational age was determined from the measurement of the fetal crown-rump length [15]. The measured NT was transformed into likelihood ratio for each trisomy using the mixture model of NT distributions [16]. The measured free ß-hCG and PAPP-A were converted into a multiples of the median (MoM) for gestational age adjusted for maternal weight, racial origin, smoking status, method of conception, parity and machine for the assays [17]. The nasal bone was assessed as being present or absent, flow across the tricuspid valve was classified as normal or regurgitant and flow in the ductus venosus was classified according to the a-wave as normal or reversed [1].

Maternal venous blood (10 mL) collected, before chorionic villous sampling, in ethylene diamine tetraacetic acid (EDTA) BD vacutainer^{TM} tubes (Becton Dickinson UK limited, Oxfordshire, UK) was processed within 15 min of collection and centrifuged at 2,000g for 10 min to separate plasma from packed cells and buffy coat (plasma 1), and subsequently at 16,000g for 10 min to further separate cell debris (plasma 2). Plasma 1 and plasma 2 (2 mL each) were divided into 0.5 mL aliquots in separate eppendorf tubes which were labeled with a unique patient identifier and stored at −80^{o}C until

subsequent analysis. Written informed consent was obtained from the women agreeing to participate in the study, which was approved by King's College Hospital Ethics Committee.

We searched our database and selected 50 consecutive cases of trisomy 21 and 50 with trisomy 18 with 2 mL of available stored plasma 2, corresponding to four tubes of 0.5 mL aliquots per case. Each one of these 100 aneuploid cases was matched with three euploid controls for length of storage of their blood samples and none of the samples were previously thawed and refrozen. Maternal blood was collected between March 2006 and August 2011. We excluded pregnancies conceived by in-vitro fertilization.

Laboratory analysis

Plasma samples (4 tubes of 0.5 mL per patient) from selected cases were sent overnight on dry ice from London, UK to the laboratory of Aria Diagnostics, Inc. in San Jose, CA. The information provided to Aria Diagnostics for each case was: patient unique identifier, maternal age, gestational age, date of blood collection and fetal sex, but not fetal karyotype. Prior to evaluation for fetal trisomy, Aria Diagnostics assessed each sample for sample volume, adequacy of labeling, and risk of contamination or sample mixing and informed us that a total of 25 samples did not meet their acceptance criteria (in 8 cases the total plasma volume after pooling of individual tubes was less than 2 mL, in 5 cases the labels on the tubes did not match the patient identifier on the file provided to the laboratory and in 12 cases there were potential issues of sample mixing or cross contamination after pooling of the individual tubes by laboratory

personnel). In 11 of these cases we had stored samples of plasma 1 (4 tubes of 0.5 mL per patient) but in 14 there was either no or insufficient plasma 1 and these were replaced with the next available cases. The samples from these 25 cases were sent to Aria Diagnostics and we were informed that all cases fulfilled the acceptance criteria of the laboratory. The 400 samples that fulfilled the acceptance criteria of Aria Diagnostics were then analyzed using their previously published technique of the DANSR assay with the FORTE algorithm [14].

Results were provided on the risk of trisomy 21 and trisomy 18 on each of the 400 cases that fulfilled the acceptance criteria to KHN who then determined the correlation between the assay results with the fetal karyotype.

Results

The characteristics of the euploid and aneuploid pregnancies are summarized in Table 1. In the aneuploid groups, compared to the euploid pregnancies, the median maternal age, median delta NT and prevalence of absent nasal bone, tricuspid regurgitation and reversed a-wave in the ductus venosus were significantly higher. In trisomy 21 serum free ß-hCG was higher and PAPP-A was lower and in trisomy 18 both free ß-hCG and PAPP-A were lower.

Risk scores for trisomy 21 and trisomy 18 were given for 397 of the 400 samples that were analyzed. In all 50 cases of trisomy 21 the risk score for trisomy 21 was $\geq$99% and the risk score for trisomy 18 was $\leq$0.01% (Figure 1). In all 50 cases of trisomy 18 the risk score for trisomy 21 was $\leq$0.01% and the risk score for trisomy 18 was $\geq$99% in 47 cases, 98.8% in one, 88.5% in one and 0.11% in one. In 3 (1%) of the 300 euploid

pregnancies no risk score was provided because there was failed amplification and sequencing. In the remaining 297 the risk score for trisomy 21 was ≤0.01% and the risk score for trisomy 18 was ≤0.01% in 295 cases, 0.04% in one and 0.23% in one. Therefore, the sensitivity for detecting trisomy 21 was 100% (50/50), the sensitivity for trisomy 18 was 98% (49/50) and the specificity was 100% (297/297).

Comment

Principal findings of this study

This nested case-control study has shown that in pregnancies at high-risk for aneuploidies chromosome-selective sequencing of cfDNA in maternal plasma obtained during the first trimester of pregnancy distinguished all cases of trisomy 21 and 98% of trisomy 18 from euploid pregnancies. The FORTE algorithm combined the risk computed from DANSR with the maternal age-related risks to estimate the patient-specific odds of trisomy versus disomy. In all cases of trisomy 21 the estimated risk for this aneuploidy was ≥99%, whereas in all euploid pregnancies and in those with trisomy 18 the risk score for trisomy 21 was ≤0.01%. In the case of trisomy 18 non-invasive testing correctly identified 98% of the cases, where the risk score for this aneuploidy was more than 88%, whereas in all euploid pregnancies and in those with trisomy 21 the risk score for trisomy 18 was less than 0.3%.

Limitations of the study

This study was based on small volumes of stored plasma samples. We provided four tubes of 0.5 mL aliquots per patient and 25 cases did not fulfill the acceptance criteria of

the laboratory. In 11 of these cases we had a further plasma sample of 2 mL and this was adequate for analysis. The remaining 14 cases with no additional stored samples were replaced with new patients. In prospective clinical studies with collection of larger volumes of blood it is likely that most of these problems will be overcome.

In 1% of the samples that were considered to be adequate for analysis there was failure to get a result. This is compatible with the 1.4% failure rate in the combined data from three previous studies utilizing MPSS [7-9]. In all these studies plasma samples were obtained from high-risk pregnancies where there is some evidence of impaired placental function, reflected for example, in low first-trimester serum PAPP-A levels. Since in pregnancies with impaired placentation the maternal plasma concentration of cffDNA is increased [18,19] the failure rate of non-invasive testing in low-risk pregnancies may be increased. It is therefore necessary to evaluate fetal DNA fraction in clinical studies in the general pregnant population.

Comparison of the findings with previous studies in the literature

Recent studies using MPSS have demonstrated that most cases of trisomy 21 can be detected from the analysis of maternal plasma cfDNA, at a very low false positive rate [8-12]. Maternal plasma was examined from a combined total of 350 trisomy 21 and 2,061 euploid pregnancies at 6-38 (median 15) gestational weeks with reported detection and false positive rates of 99% and 0.3%, respectively [8-11]. These results suggest that testing of maternal plasma cfDNA by MPSS is a high performance screening rather than a diagnostic test for fetal trisomy 21 [20]. Additionally, current MPSS based approaches are costly and have low throughput. Each sequencing

analysis run examines approximately 50 patient samples and takes several days to complete.

In this study, compared to previous publications on cfDNA, we have exclusively used samples obtained in the first trimester. This is important because in the last decade there has been a major shift from second to first trimester screening and diagnosis of aneuloidies. The use of chromosome-specific sequencing of polymorphic and non-polymorphic loci described in this study requires 10 times less DNA sequencing than MPSS approaches and can analyze approximately 750 patient samples per sequencing analysis run. This opens the possibility to a more affordable non-invasive cfDNA test for fetal trisomies 21 and 18. Other targeted approaches in development could also lead to affordable cfDNA testing [21].

Implications for practice

Ultrasound examination at 11-13 weeks' gestation allows accurate determination of gestational age, early diagnosis of major fetal malformations, diagnosis of multiple pregnancies and determination of chorionicity and ultrasound in combination with maternal serum biochemical testing provides effective screening for aneuploidies [1]. There is also increasing evidence that many pregnancy complications, including preterm birth, preeclampsia, gestational diabetes mellitus, stillbirth, fetal growth restriction and macrosomia, can now be predicted at an integrated first hospital visit at 11-13 weeks combining data from maternal characteristics and history with findings of biophysical and biochemical tests [22]. It is therefore likely that in the future such 11-13 weeks assessment will become more widespread rather than be replaced by a test for

aneuploidies.

Fetal trisomy evaluation with cfDNA testing will inevitably be introduced into clinical practice. This would be useful as a secondary test contingent on the results of a more universally applicable primary method of screening. For example, for some parents, maternal plasma cfDNA testing may be preferable to invasive testing when the first-trimester combined test suggests that their pregnancy is at very high-risk for trisomy 21. Such parents of course should be made fully aware that a positive result does not always imply that the fetus is affected and that a negative result does not imply that the fetus is euploid [20]. Indeed, in about half of the aneuploid fetuses identified by the combined test as being at high-risk for trisomy 21 the chromosomal abnormality is other than trisomy 21 [2,23].

Another population that may benefit from screening by maternal plasma cfDNA is the one identified by the combined test as being at intermediate-risk for trisomy 21, because in these cases their risk will be revised to either very high or very low, thereby making their decision in favor or against invasive testing easier [24,25].

The extent to which cfDNA analysis could also be applied as a universal screening tool for trisomy 21 in all pregnant women would depend on whether the cost becomes comparable to that of current methods of sonographic and biochemical testing. It would also be necessary to demonstrate that the observed accuracy with cfDNA testing obtained from the investigation of pregnancies at high-risk for aneuploidies is applicable to the general population where the prevalence of fetal trisomy 21 is much lower. This may well prove to be the case because the ability to detect aneuploidy with cfDNA is dependent upon assay precision and fetal DNA

percentage in the sample rather than the prevalence of the disease in the study population.

Conclusions

This nested case-control study has demonstrated that the DANSR assay with FORTE algorithm represent a promising method for accurate detection of fetal trisomy 21 and trisomy 18 from maternal blood cfDNA in the first-trimester of pregnancy. However, further research is needed to firstly, investigate the accuracy of the test in intermediate- and low-risk pregnancies, secondly, to improve the FORTE algorithm by incorporating risks derived from various biochemical and sonographic markers of aneuploidy, in addition to maternal age-related and DANSR risk, and thirdly, to expand the spectrum of aneuploidies that could be detected from analysis of maternal plasma cfDNA.

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Figure legend

Figure 1. Risk scores for trisomy 21 (left) and trisomy 18 (right) in pregnancies with trisomic (red diamonds) and euploid (blue diamonds) fetuses. The risk score for trisomy 21 was ≥99% in all 50 cases of trisomy 21 and ≤0.01% in all 297 euploid cases (left). The risk score for trisomy 18 was ≥88.5% in 49 of the 50 cases of trisomy 18 and ≤0.23% in all 297 euploid cases (right).

Table 1. Maternal and fetal characteristics in euploid and aneuploid pregnancies.

Maternal characteristic	**Euploid (n=300)**	**Trisomy 21 (n=50)**	**Trisomy 18 (n=50)**
Maternal age in years, median (IQR)	35.4 (29.9-38.5)	38.9 (34.7-41.2)**	38.0 (33.4-40.7)**
Maternal weight in Kg, median (IQR)	66.7 (60.0-76.3)	62.9 (58.3-68.3)	69.3 (60.5-78.0)
Maternal height in m, median (IQR)	163.5 (160.0-167.6)	165.1 (160.0-167.6)	165.1 (162.6-170.2)
Racial origin			
Caucasian, n (%)	268 (89.3%)	45 (90.0%)	42 (84.0%)
Afro-Caribbean, n (%)	15 (5.0%)	0	5 (10.0%)
South Asian, n (%)	9 (3.0%)	4 (8.0%)	3 (6.0%)
East Asian, n (%)	6 (2.0%)	2 (1.0%)	0
Mixed, n (%)	2 (0.7%)	0	0
Nulliparous, n (%)	87 (29.0%)	11 (22.0%)	18 (36.0%)
Cigarette smoker, n (%)	35 (11.7%)	6 (12.0%)	2 (4.0%)
Crown-rump length in mm, median (IQR)	72.6 (64.9-77-7)	71.2 (65.7-76.4)	58.5 (55.0-63.5)**
Gestation in day, median (IQR)	93.6 (89.8-96.0)	92.9 (90.2-95.4)	86.4 (84.6-89.1)**
Delta NT in mm, median (IQR)	0.66 (0.22-1.36)	1.80 (1.13-3.14)**	4.76 (2.20-6.14)**
Absent nasal bone, n (%)	18 (6.0%)	29 (58.0%)**	34 (68.0%)**
Ductus venosus reversed a wave, n (%)	22 (7.3%)	29 (58.0%)**	31 (62.0%)**
Tricuspid regurgitation, n (%)	21 (7.0%)	36 (72.0%)**	23 (46.0%)**
PAPP-A MoM, median (IQR)	0.71 (0.47-1.06)	0.55 (0.42-0.77)*	0.19 (0.11-0.26)**
β-hCG MoM, median (IQR)	1.51 (0.85-2.45)	2.54 (1.75-4.36)**	0.21 (0.12-0.38)**

Comparison between outcome groups by Mann-Whitney U-test for continuous variables and χ^2-test or Fisher's exact test for categorical variables, both with *post-hoc* Bonferroni correction. *p<0.05 **p<0.0001.

NT = nuchal translucency, MoM = multiple of median, IQR= interquartile range

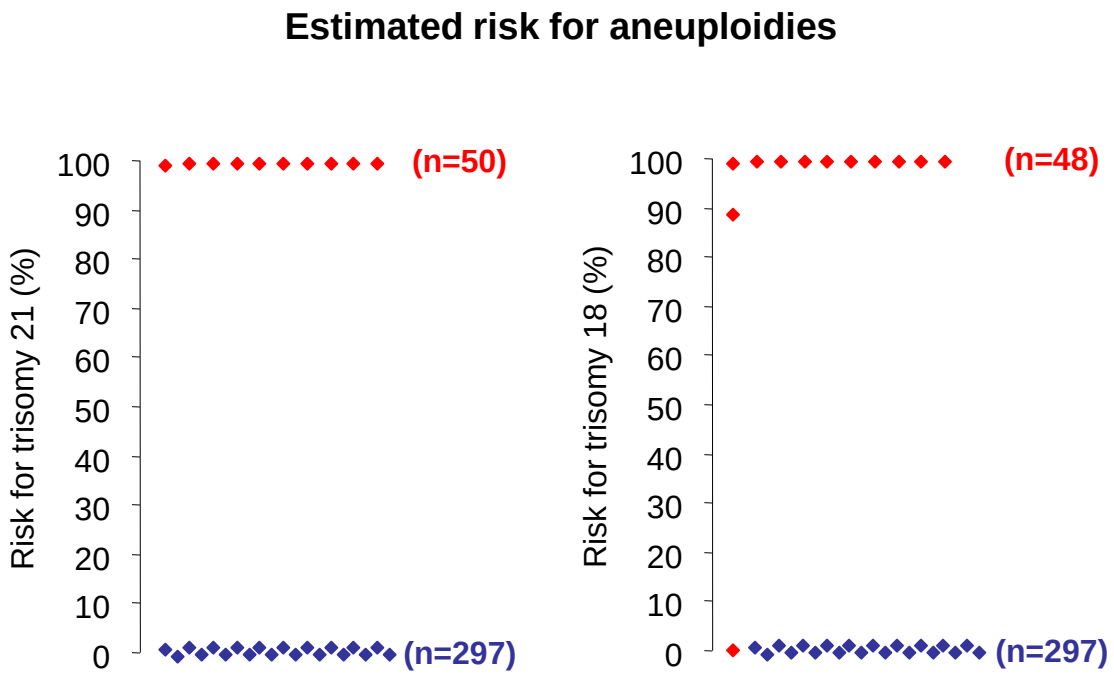
Estimated risk for aneuploidies
Risk for trisomy 21 (%)
100
90
80
70
60
50
40
30
20
10
0
(n=50)
(n=297)
Risk for trisomy 18 (%)
100
90
80
70
60
50
40
30
20
10
0
(n=48)
(n=297)

Figure 1